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**The Middle Half Genome of Interferon-Inducing Porcine Reproductive and Respiratory
Syndrome Virus Strain A2MC2 Is Essential for Interferon Induction**

Running title: The Middle Half of A2MC2 Is Required for IFN Induction

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Abbreviations: PRRSV - porcine reproductive and respiratory syndrome virus, PRRS - porcine reproductive and respiratory syndrome, IFN – interferon, nt – nucleotide, rA2MC2 – recovered PRRSV A2MC2, PAM - pulmonary alveolar macrophage, PRR - pattern recognition receptors, RLR - RIG-I-like receptors, TLR - Toll-like receptors, PAMP - pathogen-associated molecular patterns, IRF-3 - IFN regulatory factor 3, ORF - open reading frames, dsRNA - double-stranded RNA, IFA - Immunofluorescence assay, DAPI - 4'6-diamidino-2-phenylindole, SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is known to antagonize the innate immune response. An atypical PRRSV strain A2MC2 is capable of inducing synthesis of type I interferons (IFNs) in cultured cells. Here, we show that the middle half of the A2MC2 genome is needed for triggering the IFN synthesis. First, a cDNA infectious clone of this atypical strain was constructed as a DNA-launched version. Virus recovery was achieved from the infectious clone and the recovered virus, rA2MC2, was characterized. The rA2MC2 retained the feature of interferon induction in cultured cells. Infection of pigs with the rA2MC2 virus caused viremia similar to that of the wild type virus. Chimeric infectious clones were constructed by swapping genomic fragments with a cDNA clone of a moderately virulent strain VR-2385 that antagonizes IFN induction. Analysis of the rescued chimeric viruses demonstrated that the middle two fragments, ranging from nt4545 to nt12709 of the A2MC2 genome, were needed for the IFN induction, whereas the chimeric viruses containing any one of the two A2MC2 fragments failed to do so. The results and the cDNA infectious clone of the IFN-inducing A2MC2 will facilitate further study of its biology, ultimately leading towards the development of an improved vaccine against PRRS.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) infection of pigs has a high economic impact on swine production across the world, and has resulted in an estimated \$664 million loss per year in the United States alone [1, 2]. PRRSV is a single-stranded positive-sense RNA virus belonging to the family *Arteriviridae*, order *Nidovirales* [3]. There are two PRRSV genotypes: Type 1 (European) and Type 2 (North American), which are classified into two species in the genus *Porartevirus*: *PRRSV-1* and *PRRSV-2* in the new taxonomy [4, 5]. The genome of PRRSV is around 15 kb in length and contains at least ten open reading frames (ORFs). ORF1a and ORF1b occupy two thirds of the viral genome and encode non-structural proteins that are needed for viral replication, while ORFs 2-7 encode structural proteins. PRRSV mainly targets pulmonary alveolar macrophages (PAMs) during acute infection of pigs [6]. MARC-145 cells, derived from a monkey kidney, are generally used for PRRSV propagation *in vitro* [7].

Host innate immune responses play a critical role against early viral infection. The pattern recognition receptors (PRR) for RNA viruses include RIG (retinoic-acid-inducible gene)-I-like receptors (RLRs) and Toll-like receptors (TLRs) [8, 9]. Stimulation of RLR and TLR signaling pathways leads to activation of IFN regulatory factor 3 (IRF-3), IRF-7 and NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), followed by induction of type I interferons (IFNs) (i.e. IFN- α and β) and expression of inflammatory cytokines. Type I IFNs are critical to innate immunity against viral infections and play an important role in activation of the adaptive immune response [10, 11].

PRRSV appears to inhibit synthesis of type I IFNs *in vivo* [12-14] and *in vitro* [12, 15, 16]. Recovered virus from an infectious clone of a high-pathogenic PRRSV isolate was reported

to induce IFNs in infected pigs, which may be because of its significant higher-level replication (100-1000 fold) and induced more severe inflammatory response than VR-2332 [17]. An atypical PRRSV strain A2MC2 was discovered to induce high level IFNs in cultured cells, whereas other strains tested, including PRRSV VR-2332, Ingelvac[®] PRRS MLV (Boehringer Ingelheim, Inc; hereinafter referred as MLV vaccine strain), and VR-2385, antagonize IFN induction [18]. Inoculation of pigs with the A2MC2 virus leads to earlier onset and higher virus-neutralizing antibodies than the MLV vaccine strain [19]. Neutralizing antibodies against PRRSV confer protection of pregnant sows against reproductive failure induced by virulent strain challenge [20]. Passive transfer of PRRSV-neutralizing antibodies to young weaned pigs blocks PRRSV viremia after challenge [21].

The interferon induction by strain A2MC2 sustains serial passaging of the virus in MARC-145 cells for 90 passages for attenuation, as the A2MC2-P90 induces IFNs similarly to the wild type virus [22]. This high-passaged virus, A2MC2-P90, is avirulent and induces higher virus-neutralizing antibodies than the MLV vaccine strain. The interferon induction of A2MC2 and its ability to induce high levels of neutralizing antibodies indicate that this virus carries unique feature in its genomic sequence, which might correspond to pathogen-associated molecular patterns (PAMP). The PAMP is prone to be recognized by host PRR and remains intact in the avirulent A2MC2-P90 virus.

In the present study, the objective was to construct a cDNA infectious clone of A2MC2 and to study the genomic source of the interferon induction. A DNA-launched infectious clone was constructed, and virus recovery was achieved. The recovered virus maintained the feature of IFN induction in cultured cells. When the recovered virus was tested in the pig model, it caused pathology similar to that of the wild type virus. Chimeric clones of A2MC2 with moderately

96 virulent PRRSV strain VR-2385 that does not induce IFNs were constructed. Analysis of the
97 recovered chimeric viruses demonstrated that the middle half of the A2MC2 genome is essential
98 for the interferon induction.

99

RESULTS

Construction of cDNA infectious clone of atypical PRRSV strain A2MC2 and determination of the growth property of the recovered virus

A cDNA infectious clone of strain A2MC2 was constructed as a DNA-launched version (Fig. 1a). Sequences of hammerhead ribozyme and hepatitis delta virus ribozyme were added at the 5' and 3' terminus, respectively, of the cDNA of A2MC2. The full-length PRRSV sequence in the resulting plasmid pCAGEN-A2MC2-Rz was confirmed by DNA sequencing. MARC-145 cells were transfected with the pCAGEN-A2MC2-Rz plasmid to recover virus. The transfected cells were harvested four days after transfection and supernatant of the cell lysate was passaged in fresh cells. Typical cytopathic effect of PRRSV was visible 48 h post inoculation (hpi), and the virus proliferation was verified by IFA with an N-specific monoclonal antibody (Fig. 1b). Partial DNA sequencing of the progeny virus confirmed they were derived from the infectious clone.

The rA2MC2 virus was propagated in MARC-145 cells for a multi-step growth curve and a plaque assay. The virus yields for the cells with inoculum at an MOI (multiplicity of infection) of 0.01 peaked at 72 hpi (Fig. 2a). The virus yields for the cells inoculated with an MOI of 0.01 were higher than the cells inoculated with an MOI of 0.1 and 1.0. The virus yields for the cells inoculated with an MOI of 1.0 decreased along with time extension. The virus yields for the cells inoculated with an MOI of 0.1 were similar to those of parental wild type A2MC2 virus in the same amount of inoculum (Fig. 2a).

A plaque assay was conducted in MARC-145 cells to compare the rA2MC2 and its parental A2MC2 virus. Both parental A2MC2 and rA2MC2 had similar plaque sizes, 3-4 mm in

diameter (Fig. 2b). The results indicate that the rA2MC2 virus had a growth property similar to its parental virus.

The recovered A2MC2 virus induces interferon synthesis

As the wild type A2MC2 induces interferon production in cultured cells [18], we tested whether the rA2MC2 virus kept the feature of IFN induction. The supernatant of rA2MC2-infected MARC-145 cells was used for an interferon bioassay in Vero cells. Results showed that NDV-GFP replication was inhibited in the Vero cells treated with the rA2MC2 supernatant diluted up to 1:16, similar to the wild type A2MC2 (Fig. 3a). This suggests that rA2MC2 induced production of interferons, which led to the suppression of NDV replication in the Vero cells, in a similar capacity to the wild type A2MC2 virus.

Expression of interferon-stimulated genes (ISG) was also determined to confirm the effect of the treatment of Vero cells. Compared with the mock-treated cells, the cells treated with supernatant samples of rA2MC2 and parental wild type A2MC2 at 24 hpi had ISG15 transcript level increased by 64 and 81-fold, respectively (Fig. 3b). There is no significant difference between the ISG15 levels in cells treated with rA2MC2 or wild type A2MC2 supernatants. STAT2 and RIG-I are also known to be upregulated by type I IFNs [18, 22]. The protein levels of STAT2 and RIG-I in the rA2MC2-infected MARC-145 cells at both 24 and 48 hpi were upregulated (Fig. 3c). The result demonstrated that rA2MC2 kept the feature of IFN induction.

PAMs are the major target cells for PRRSV infection *in vivo*. We inoculated primary porcine PAMs with the rA2MC2 virus and found that the virus replicated in PAMs and induced IFNs (data not shown). This indicates the recovered virus is similar to the wild type A2MC2 virus in inducing IFN synthesis in the cultured cells.

Pig study

An animal experiment was conducted by inoculating 3-week-old PRRSV-negative piglets with the rA2MC2 virus. The pigs were euthanized at 14 days post-infection (DPI). Compared with the pigs inoculated with parental A2MC2 virus, the pigs infected with the rA2MC2 virus had similar lesion scores, though there were some low responders (Fig. 4a).

Real-time PCR was conducted to determine PRRSV RNA in serum samples of 14 DPI. The average PRRSV RNA levels for the pigs infected with the rA2MC2 and the parental A2MC2 virus were 4.55 and 4.46 log₁₀ copies/ml, respectively (Fig. 4b). There was no significant difference in the PRRSV RNA levels between the two groups. These results suggest that the rA2MC2 virus has similar virulence and replication kinetics in pigs compared to its parental A2MC2 virus.

Construction of chimeric cDNA infectious clones and determination of the growth property of the recovered chimeric virus

To determine the genetic source of A2MC2 virus for interferon induction, we constructed three chimeric A2MC2 infectious clones by genomic fragment swapping with pIR-VR2385 (Fig. 5a). VR-2385 does not induce IFNs [18] and the pIR-VR2385 was robust in virus recovery [23]. The cDNA sequence of the viral genome in pIR-VR2385 is shorter than the A2MC2 sequence due to deletion in nsp2 [23]. So the nucleotide positions of the three restriction enzymes are different from A2MC2 cDNA (Fig. 5a). The F2, F3, and F1-F3 fragments in pIR-VR2385 were replaced with their counterparts of A2MC2, resulting in chimeric clones: pIR-VR2385/A2F2, pIR-VR2385/A2F3, and pIR-VR2385/A2F123. MARC-145 cells were transfected with these chimeric infectious clones as well as pIR-VR2385 and pIR-A2MC2 plasmids for virus recovery separately. The virus recovery was successful from all the clones.

The chimeric viruses were subjected to growth property assay in MARC-145 cells. The virus yields for the cells inoculated with the rA2F2 and rVR2385 from 48 to 96 hpi were similar (Fig. 5b). The virus yields for the cells inoculated with the rA2MC2 and rA2F3 were similar, approximately 0.5-1 log lower than rA2F2 and rVR2385 at 72 and 96 hpi. All the viruses had similar yields at 48 hpi. The yields for the rA2F123 virus at 72 and 96 hpi were lower than all the other four viruses. The results indicate that the chimera rA2F2 is similar to rVR2385 in replication rate, chimera rA2F3 is similar to rA2MC2, and chimera rA2F123 has a lower replication rate than both rVR2385 and rA2MC2.

A plaque assay was conducted in MARC-145 cells to compare the chimeric viruses. All the chimeric viruses had similar plaque sizes, 2-4 mm in diameter (Fig. 5c).

The middle half of the strain A2MC2 genome is essential for interferon induction

For interferon bioassay, the culture supernatants of MARC-145 cells infected with the chimeric viruses were used to treat Vero cells. Results showed that NDV-GFP replication was inhibited in the Vero cells treated with the culture supernatants of rVR/A2F123 and rA2MC2 virus in a dilution up to 1:16 (Fig. 6a). However, treatment with the culture supernatants of rVR/A2F2, rVR/A2F3, and rVR2385 viruses had a minimal effect on the NDV-GFP replication when compared to the mock-treated cells. A2MC2 has the same sequence in fragment 1 (nt1-4544) as VR-2332, and the latter does not induce IFNs in MARC-145 cells [18]. So both fragments 2 and 3 (nt4545-12709) are critical for the IFN induction.

To confirm this observation, immunoblotting to determine the RIG-I protein level in MARC-145 infected with the chimeric viruses was conducted. Compared with the mock-infected cells, the MARC-145 cells infected with rVR/A2F123 chimeric virus at 48 hpi had higher RIG-I protein level, whereas the cells infected with rVR/A2F2 and rVR/A2F3 chimeric viruses had a

190 slight reduction (Fig. 6b). The rVR2385-infected cells had lower RIG-I level than the mock-
191 infected cells. This confirmed that the rVR/A2F123 chimeric virus induced interferon production
192 in MARC-145 cells and the presence of both fragments 2 and 3 of the A2MC2 genome is
193 required for the sensing by host pattern recognition receptors.

DISCUSSION

Although it has been nearly 30 years since the first reports of PRRS, broadly effective vaccines against PRRSV infection are still not available due to the genetic diversity of PRRSV isolates and apparent lack of cross-protection between isolates [2]. It is known that PRRSV strains generally antagonize interferon synthesis [14, 18, 24]. The effect of PRRSV replication on IFN induction appears to be variable among different strains and different cell types. PRRSV field isolates have variable suppressive effect on IFN- α induction in cultured PAM cells [16]. The interferon induction by PRRSV strain A2MC2 is a unique feature of this virus. Considering the importance of interferons in activating the adaptive immune response [11, 25], this feature is desirable in vaccine development against PRRSV. Type I IFNs have an important role in the differentiation of CD4⁺ and CD8⁺ T cells [11, 25]. This study identified that the middle half of the A2MC2 genome is pivotal for IFN induction by chimeric infectious clone analyses. The rescued A2MC2 virus has the capability of IFN induction in cultured cells like its parental strain. Treatment of Vero cells with the culture supernatants from rA2MC2-infected cells induced expression of ISG15, RIG-I, and STAT2, which indicates the activation of IFN signaling. The expression of the interferon-activated genes was confirmed at both the RNA and the protein levels. These results suggest that the genetic feature of A2MC2 is maintained in the cDNA infectious clone.

The recovered rA2MC2 virus maintained the feature of IFN induction in cultured cells. The rA2MC2 virus has growth properties in terms of multi-step growth and plaque formation similar to its parental virus. The cells infected with rA2MC2 inoculum at an MOI of 0.01 had higher viral yields than the cells inoculated with an MOI of 0.1 and 1.0, which indicates less inoculum leads to more efficient virus replication. This result is also consistent with the feature

of IFN induction. The lower the inoculum, the less IFN induction in the cells and the weaker the antiviral response the cells would mount. The virus in the cells at low level would have a better chance to replicate to high level without triggering a rigorous antiviral response than high level of virus in the initial inoculum.

The rA2MC2 virus led to pathology and viremia in pigs similar to its parental virus, though the lung lesion scores were numerically variable among the pigs infected. This is possible due to the variation of individual pigs used in this study, some of which were low responders. This is also indicated by their similar viremia levels. The rA2MC2 virions are expected to be a more homogenous population than the wild type virus though the latter was plaque purified [18].

The chimeric cDNA clones of A2MC2 and VR-2385 were constructed. Similar growth trend and yields from 48 to 96 hpi were observed between the rA2F2 and rVR2385, as well as between the rA2F3 and rA2MC2. All the five recovered viruses have similar yields at 48 hpi. The rA2F123 has similar yields to rVR2385, but lower yields than the other four viruses at 72 and 96 hpi, which may be because rA2F123 induces IFNs. The yields of the rA2MC2 virus was slightly higher than rA2F123 in the two late time points when the cells were inoculated at this amount of inoculum. It is not known whether the fragment 4 that are different between these two recovered IFN-inducing viruses contributes to the variation in late virus replication.

The chimeric clones of A2MC2 and VR-2385 were used to study the genomic source of the IFN induction. Since the nucleotide sequence of A2MC2 fragment 1 (nt1-4544) is the same as VR-2332 and the latter antagonizes IFN induction [18], the fragment 1 has no pivotal role in the A2MC2 induction of IFNs. Thus, the genomic source for the IFN induction must be from the middle two fragments. The results from analyzing the chimeric viruses demonstrated that both fragment 2 and 3 (nt4545-12709) are required for IFN induction. The sequence of nt4545-12709

in the A2MC2 genome encodes proteins of C-terminal 126 amino acid residues of nsp2, nsps 3-12, and N-terminal 213 residues of GP2a. Among these PRRSV nsps, nsp2, nsp4, and nsp11 are reported to inhibit IFN induction [14, 26]. A2MC2 has the identical nsp2, nsp4, and nsp11 as VR-2332 or MLV [18]. Thus, lack of inhibition of IFN induction by these proteins is not possible.

Our results indicate that the middle half genome of A2MC2 contributes to the PAMP recognition by host PRR. RIG-I and MDA5 are the PRR to sense viral RNA in the cytoplasm. RIG-I preferentially recognizes the 5' terminus of the virus RNA, including both the genomic and subgenomic RNA for PRRSV. A prerequisite for RIG-I recognition is the presence of 5' diphosphate or triphosphate [27, 28]. Conversely, MDA5 recognizes an internal duplex structure of long double-stranded RNA (dsRNA) [29, 30]. RIG-I was previously reported to be the PRR interfered by PRRSV to antagonize IFN induction [31]. For IFN induction by strain A2MC2, the role of RIG-I is unknown. Presumably, dsRNA is formed during PRRSV replication, and the middle half of the A2MC2 genome confers the internal duplex structure for MDA5 recognition. This is consistent with our observation that A2MC2 replication is needed for the IFN induction as UV-inactivated A2MC2 virus cannot trigger IFNs [18]. We speculate that many PRRSV strains like VR-2332 and VR-2385 that antagonize IFN induction may escape the MDA5 recognition by formation of a different internal duplex structure.

Our data indicate that both fragments 2 and 3 (nt4545 to nt12709) of A2MC2 are pivotal for the possible internal duplex structure for MDA5 recognition. Replacement of either F2 or F3 in the cDNA clone of VR-2385 with the corresponding fragment of A2MC2 failed to confer the chimeric viruses the capability to induce IFNs. In addition, the chimeric viruses from the F2 or F3 chimeric clones were unable to evoke elevation of RIG-I. In contrast, the chimeric virus

rA2F123 containing both the F2 and F3 of A2MC2 induced IFNs and triggered the elevation of RIG-I protein level. The result indicates that the A2MC2 fragment 4 (nt12710-3'terminus) covering ORFs 3-7 appears not to play a decisive role in the IFN induction. A2MC2 ORF2 (nt12073-12843) overlaps with the 3'terminus of F3 and the 5'terminus of fragment 4. It is not known whether the ORF2 sequence contributes to the potential internal duplex structure for MDA5 recognition. The requirement of both fragment 2 and 3 in IFN induction might be coincidental. Further study is needed to define the minimum sequence and/or nucleotides that are required for the IFN induction.

Though the fragment 1 is assumed to have no pivotal role in the IFN induction by strain A2MC2, other studies have shown three proteins encoded by this part of the genome, nsp1 α , nsp1 β , and nsp2, involve in PRRSV inhibition of IFN induction [14, 24, 32]. Reversal of the inhibition leads to improvement of IFN induction. Site-directed mutagenesis of R128 and R129 of nsp1 β reduced its inhibition of IFN induction and led to improvement of the innate and adaptive immune responses by the mutant virus [33]. A synthetic PRRSV strain that was prepared on the basis of a consensus genome from alignment of 59 full-length genomes is shown to induce IFNs [34] and elicits heterologous protection [35]. The IFN induction phenotype of the synthetic PRRSV was mapped to the 3.3 kb genome encoding nsp1 α , nsp1 β , and N-terminal part of nsp2 [34]. Our data indicates that A2MC2 has a unique mechanism to trigger IFN synthesis, which is presumed to begin before the nsps are able to mount sensible suppression. This presumption is in consistent with our result that replication kinetics of A2MC2 is inverse proportional to the inoculum MOI. This mechanism is stable during serial passaging for 90 times in cultured cells since the A2MC2-P90 maintains the feature of IFN induction [22].

In conclusion, the middle half genome of strain A2MC2 is pivotal for its IFN induction.

286 The DNA-launched cDNA infectious clone of A2MC2 was constructed, and the recovered virus
287 carries the unique feature of interferon induction in cultured cells. Further study using the
288 A2MC2 cDNA infectious clone is warranted for examination of the precise mechanism of IFN
289 induction and development of an improved vaccine against PRRS.

MATERIALS AND METHODS

Cells and viruses

Vero (ATCC CCL-81) and MARC-145 [7] cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS). CRL2843 cells (porcine macrophages, ATCC) were cultured in RPMI1640 medium supplemented with 10% FBS. Primary PAM cells were prepared from 4-8-week-old piglets and cultured in RPMI1640 medium supplemented with 10% FBS [36].

PRRSV strains VR-2385 (passage 15) and A2MC2 (passage 10) were propagated and titrated in MARC-145 cells. Virus yields were titrated by 10-fold serial dilutions and presented as the median tissue culture infectious dose (TCID₅₀) [37]. Newcastle disease virus strain LaSota carrying the gene of green fluorescence protein (NDV-GFP) was propagated in embryonated eggs and titrated in Vero cells [38].

RNA isolation, reverse transcription, PCR and real-time PCR

Total RNA was isolated using the TRIzol® LS Reagent (Thermo Fisher Scientific Inc., Waltham, MA) following the manufacturer's instructions. Reverse transcription and PCR (RT-PCR) and real-time PCR (RT-qPCR) were conducted to amplify cDNA for cloning or to determine PRRSV RNA levels [36, 39]. To normalize the total input RNA, ribosomal protein L32 (RPL32) RNA was measured. Specifically, the analysis of the relative transcript levels was performed by normalization of RPL32 in comparison with controls [40].

Construction of cDNA clone of PRRSV strain A2MC2

The strategy to construct the cDNA clone of A2MC2 (passage 10) is illustrated in Fig. 1a [41]. PCR was done to amplify four fragments spanning the full-length cDNA of strain A2MC2 genomic RNA. Primers used in the PCR are listed in Table 1. The unique restriction sites FseI

(nt4545), PmeI (nt7692) and BsrGI (nt12709) in the A2MC2 genome were used to assemble the cDNA clone. SphI and PacI were also used to clone the cDNA into the target vector pCAGEN, which was a gift from Connie Cepko (Addgene plasmid # 11160) ([42]. A stuffer sequence containing these restriction sites was designed and ligated into pCAGEN at EcoRI/XhoI sites to generate the pCAGEN-Stuffer. The PCR products digested with the restriction enzymes were ligated into the pCAGEN-Stuffer vector in the following order: F1, F4, F3 and F2 to generate pCAGEN-A2MC2. DNA sequencing was done to confirm the cloned fragments. For insertion of a hepatitis delta virus ribozyme to the 3' end cDNA of the virus genome, two oligos A2-3endRiboF and A2-3endRiboR were annealed and digested with PacI before ligation into pCAGEN-A2MC2 that was prepared with PacI digestion. DNA sequencing was done to confirm the addition, orientation and correct sequence of the two ribozymes in the recombinant pCAGEN-A2MC2-Rz.

Correction of point mutations in the cDNA clone was done with Thermo Scientific™ Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific). The cDNA clone of PRRSV VR-2385, pIR-VR2385-CA, was a gift from Xing-Jin Meng [23] and used for fragment swapping with A2MC2 cDNA to generate chimeric clones and the pIR-A2MC2 plasmid. The rA2MC2 virus (passage 6) was used in experiments in this study.

Interferon bioassay

The presence of interferons in the culture supernatant samples was determined as described previously [18]. Briefly, culture supernatant samples from PRRSV-infected MARC-145 cells were diluted and added to Vero cells. After overnight incubation, the Vero cells were inoculated with NDV-GFP, as it is sensitive to IFN-induced antiviral effect. Fluorescence microscopy was conducted 24 h after NDV inoculation to observe GFP-positive cells.

Immunofluorescence assay (IFA)

PRRSV propagation in MARC-145 cells on coverglass was detected with IFA using an N-specific monoclonal antibody EF11 [43]. DyLight™ 488 conjugated goat anti-mouse IgG (Rockland Immunochemicals Inc., Limerick, PA) was used to detect the EF11 binding to the N protein in the infected cells. SlowFade Gold antifade reagent containing 4'6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) was used for mounting the coverglass onto the slide before observation under fluorescence microscopy.

Western blotting

Total proteins in the cell lysate samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting as described previously [44]. Antibodies against STAT2, RIG-I, and GAPDH (Santa Cruz Biotechnology, Inc., Dallas, TX) and horseradish peroxidase-conjugated secondary antibodies (Rockland Immunochemicals Inc.) were used in this study.

Plaque assay

Plaque assay in MARC-145 cells was done to compare the recovered virus with its parental virus [18, 22]. PRRSV was diluted to 10 and 100 TCID₅₀ per ml and added to the monolayer cells. After 2 h incubation, the inoculum was removed and 0.5% agarose overlay containing complete growth medium was added onto the cells. Plaques were stained with neutral red and observed after overnight incubation.

DNA Sequencing

DNA sequencing was performed with ABI Prism 3130 Genetic Analyzer (Thermo Fisher Scientific). Sequence assembly and analysis was done with LaserGene Core Suite (DNASTAR

Inc., Madison, WI). The GenBank accession number of the cDNA sequence of A2MC2 genomic RNA is JQ087873.

Animal study

The animal study was approved by the Institutional Animal Care and Use Committees (IACUC) of Iowa State University and the University of Maryland and according to relevant guidelines and policies for the care and use of laboratory animals. Three-week-old PRRSV-negative piglets were randomly divided into three groups with 4 pigs in each group. The piglets in groups 1 and 2 were inoculated with 1 ml of wild type A2MC2 (passage 10) and rA2MC2 (passage 6), respectively, at 10^5 TCID₅₀/ml via intranasal inoculation (I.N.), while group 3 was mock-infected with phosphate-buffered saline (PBS) pH7.2. The pigs were observed daily. Blood samples were collected prior to inoculation and at 14-day post-infection (DPI).

The pigs were euthanized on DPI14 by pentobarbital overdose (FATAL-PLUS, Vortech Pharmaceuticals, LTD. Dearborn, MI). Visible macroscopic lung lesions were scored and recorded as previously described [45, 46].

Statistical analysis

Differences between treatment and control samples were assessed by the *t*-test (Mann-Whitney test (nonparametric)) in GraphPad Prism. A two-tailed *P*-value of less than 0.05 was considered statistically significant.

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383 **CONFLICT OF INTEREST**

384 None.

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511 Table 1. Primers used in this study

Primer*	Sequence (5' to 3') [†]
A2HeadRiboF1	ACAT <i>GCATG</i> CGGCGCGCCACATCATCTGATGAGTCCGTGAGGACGAAACGGTACCCG
A2HeadRiboF2	CGTGAGGACGAAACGGTACCCGGTACCGTCATATGACGTATAGGTGTTGGCTCTATGC
A2-4618R	CTGGGCGACCACAGTCCTA
A2-4095F	TGGCTTTTGCTGTTGGTCTGTTC
A2-8077R	GAGCGTCGCCGCGCCTAATGTC
A2-7255F	GGATGAGGACCGTTTGAATAAG
A2-13200R	CCGCCGTCGACTTGATGTTGGTAA
A2-11936F	GTGAGGACTGGGAGGATTACA
A2-3endR	GTCTTTAATTAAGTACTAGTTTTTTTTTTTTTTTTTTTTTTTAATTTTCG
A2-3endRiboF	CCTTAATTAAGGCCGGCATGGTCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATTCC GAG
A2-3endRiboR	GGTTAATTAAGTCCCATTCGCCATTACCGAGGGGACGGTCCCCTCGGAATGTTGCCAG CCG
StufferF	AATTCGCATGCATAGCCGGCCGGCCTACAGCGTTTAAACGTCCGATGTACACCTGACTTA ATTAAC
StufferR	TCGAGTTAATTAAGTCAGGTGTACATCGGACGTTTAAACGCTGTAGGCCGGCCGGCTATG CATGCG

512

513 *The primers were designed on the basis of the PRRSV A2MC2 cDNA sequence
514 (GenBank accession number JQ087873).

515 [†]Italicized letters denote target sites of restriction enzymes used in the study.

516

517

FIGURE LEGENDS

Fig. 1. Construction of a cDNA infectious clone of PRRSV strain A2MC2. (a) Schematic illustration of the strategy for the cloning of A2MC2 cDNA into pCAGEN vector. F1, F2, F3, and F4 denote four fragments amplified from cDNA of A2MC2. The numbers 4545, 7692, and 12709 above the insert indicate nucleotide positions of the restriction enzymes FseI, PmeI and BsrGI in the cDNA of A2MC2 (GenBank accession number JQ087873). The lower four lines indicate PCR amplified fragments. The five restriction enzymes used to assemble the full-length cDNA are indicated above each fragment. H-Rz: hammerhead ribozyme. D-Rz: hepatitis delta virus ribozyme. (b) Immunofluorescence assay (IFA) of MARC-145 cells infected with recovered virus from the cDNA infectious clone of A2MC2 (rA2MC2).

Fig. 2. Growth property of the rA2MC2 virus. (a) Multi-step growth curve of the rA2MC2 virus. MARC-145 cells were infected with rA2MC2 virus at an MOI of 1, 0.1, and 0.01, respectively. Culture supernatants were collected daily for virus yield titration. The virus titers (\log_{10} TCID₅₀/ml) were determined in MARC-145 cells. Error bars represent standard errors of three repeated experiments. Wild type parental A2MC2 virus was included as a control (0.1 MOI WT). (b) Plaque assay of rA2MC2 and parental A2MC2 virus in MARC-145 cells. The bars in the images correspond to 10 mm.

Fig. 3. The rA2MC2 virus induces type I interferons in MARC-145 cells. (a) Interferon bioassay in Vero cells. Cell culture supernatants from rA2MC2-infected MARC-145 cells collected at 24 hpi were diluted and added to Vero cells. 12 h later, the cells were infected with NDV-GFP. Fluorescence microscopy was conducted 24 h after NDV-GFP inoculation. Culture supernatant from A2MC2-infected cells and IFN- α at 1000 U/ml were included as controls. (b) Activation of ISG15 expression in infected MARC-145 cells (24 hpi) detected by real-time PCR. “***” denotes

a significant difference compared to the mock-infected cells ($P < 0.01$). (c) The increase of STAT2 and RIG-I protein levels in rA2MC2-infected MARC-145 cells determined by Western blotting. Sample of cells infected with parental A2MC2 were included as a control.

Fig. 4. Pig test of the rA2MC2 and its parent virus. Four pigs from each group were euthanized at 14 days post infection (DPI). Mock-infected pigs (PBS) were included as controls. (a). Gross lung lesion scores. Median values are shown. Error bars represent standard errors of the scores among the four pigs in each group. NS: no significant difference. (b). Viremia on DPI14 detected by RT-qPCR.

Fig. 5. Chimeric cDNA infectious clones and growth property of the chimeric viruses. (a) Schematic illustration of the strategy for the construction of chimeric cDNA infectious clones via fragment swapping between PRRSV strains VR-2385 and A2MC2. The numbers above pIR-VR2385 denote nucleotide positions in the VR-2385 genome. The restriction enzymes used for the fragment swapping are indicated below the pIR-VR2385 box. The shaded boxes indicate fragments from strain A2MC2. H-RZ: hammerhead ribozyme; F1 to F4: fragment 1 to 4; P(A): poly(A); D-RZ: hepatitis delta virus ribozyme; pIR-VR/A2F2: pIR-VR2385/A2F2. (b) Multi-step growth curve in MARC-145 cells infected with the virus at an MOI of 0.01. The virus titers (\log_{10} TCID₅₀/ml) were determined in MARC-145 cells. rVR2385: recovered virus from the cells transfected with pIR-VR2385; rA2F2: recovered chimeric virus from pIR-VR/A2F2; rA2F3: recovered chimeric virus from pIR-VR/A2F3; rA2F123: recovered chimeric virus from pIR-VR/A2F123; rA2MC2: recovered virus from pIR-A2MC2. (c) Plaque assay in MARC-145 cells. The bars in the images correspond to 10 mm.

Fig. 6. The middle half of A2MC2 genome is essential for the interferon induction. (a) Interferon bioassay in Vero cells. Cell culture supernatants from MARC-145 cells infected with the

564 chimeric viruses were collected and used to treat Vero cells at indicated dilutions. rVR:
565 rVR2385. (b) The increase of RIG-I protein level in MARC-145 cells infected with rA2F123
566 chimeric virus determined by Western blotting. Samples of cells infected with rVR2385 and
567 parental A2MC2 were included as controls.

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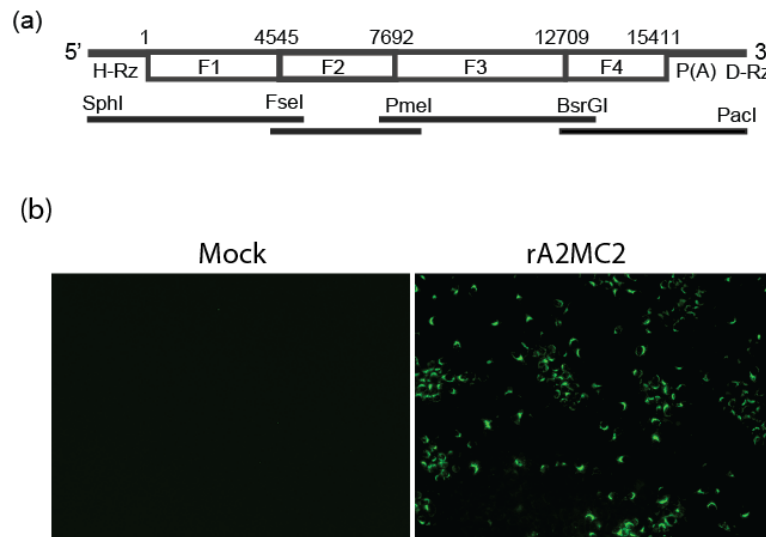


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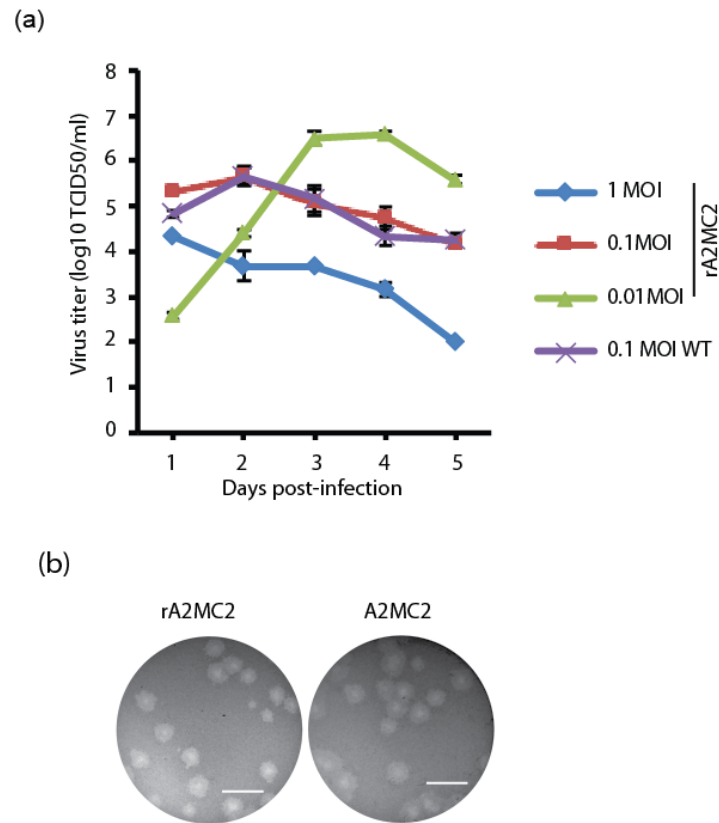


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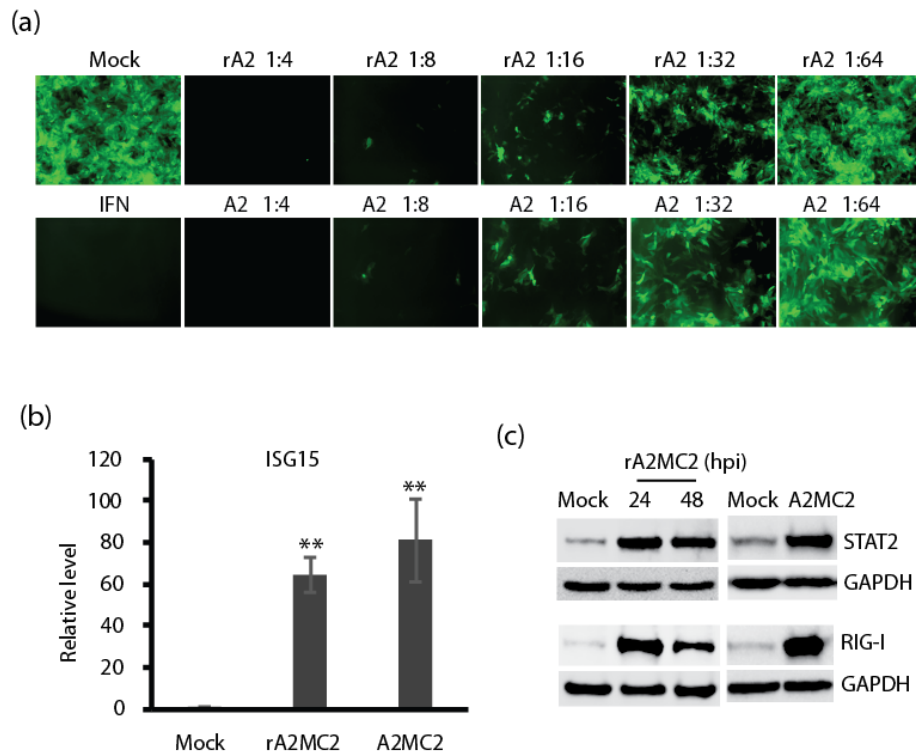


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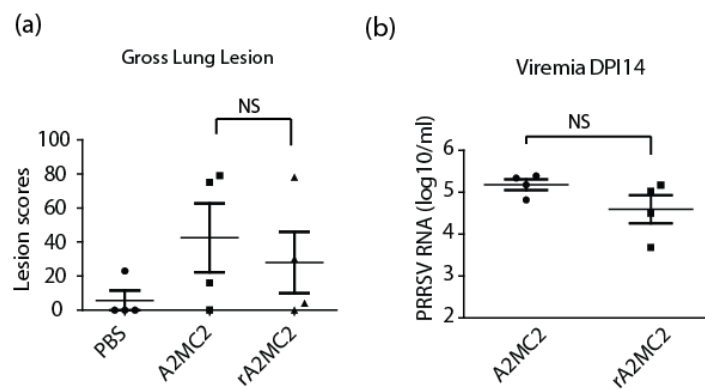


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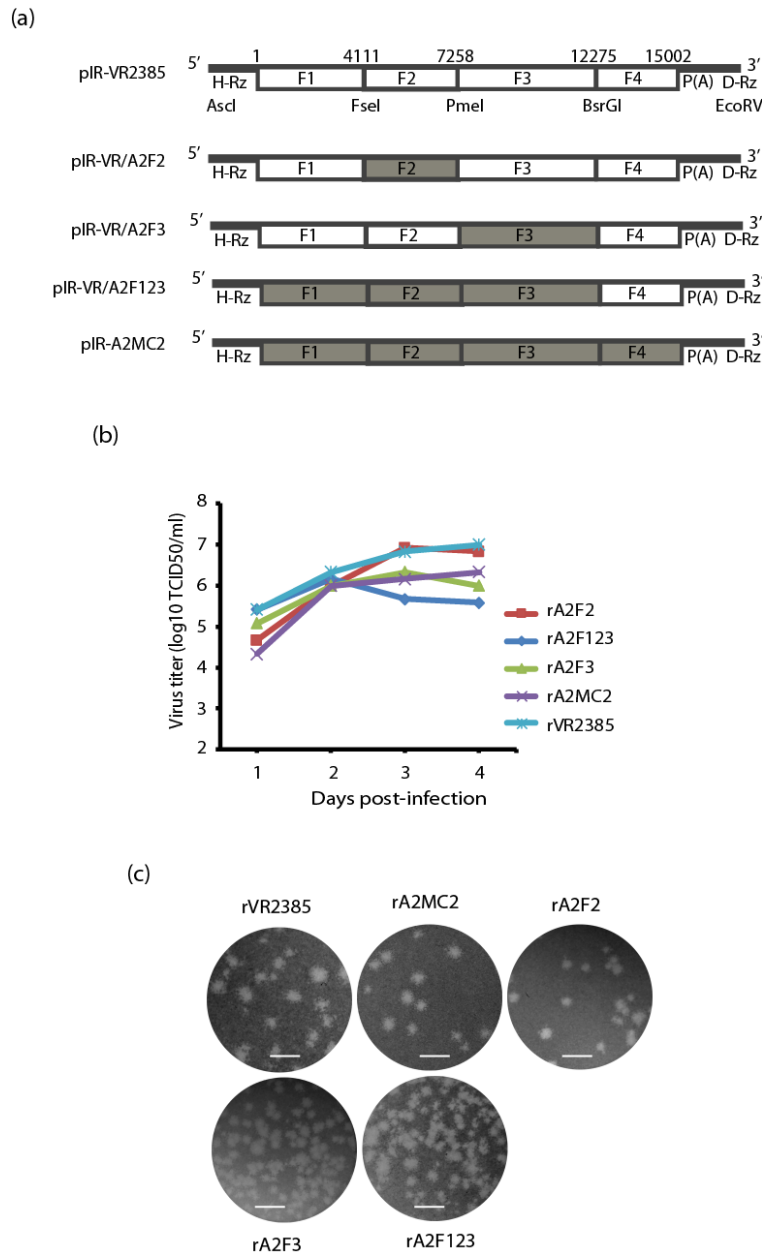


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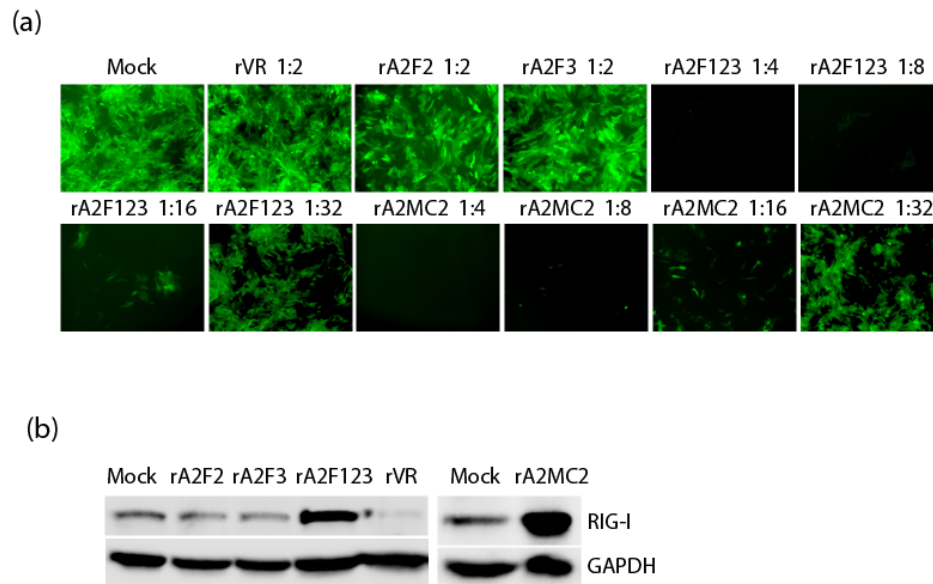


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